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Exocellular Esterase and Emulsan Release from the Cell Surface of *Acinetobacter calcoaceticus*

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An esterase activity has been found, both in the cell-free growth medium and on the cell surface of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1. The enzyme catalyzed the hydrolysis of acetyl and other acyl groups from triglycerides and aryl and alkyl esters. Emulsan, the extracellular heteropolysaccharide bioemulsifier produced by strain RAG-1, was also a substrate for the enzyme. Gel filtration showed that the cell-free enzyme was released from the cell surface either emulsan free or associated with the bioemulsifier. The partially purified enzyme was found to interact specifically with the esterified fully active emulsan, but not with the deesterified polymer. A role for esterase in emulsan release from the cell surface was indicated when the enzyme was preferentially depleted from the cell surface under conditions in which emulsan was not released. Such cells lost the capacity to release the biopolymer.

Bacterial exopolysaccharides have been the subject of extensive research regarding their chemical and physical properties, production, and numerous applications (2, 5, 7, 9, 11, 19, 21; D. L. Gutnick, E. Rosenberg, and Y. Shabtai, U.S. patent 4,234,689, 1980; D. L. Gutnick and E. Rosenberg, U.S. patent 4,276,094, 1981). One example of such materials is emulsan (molecular weight, 10^6), an amphipathic extracellular heteropolysaccharide produced by *Acinetobacter calcoaceticus* RAG-1 (14–16). The polysaccharide backbone of this polyanionic bioemulsifier consists of *N*-acetyl-D-galactosamine and an *N*-acetyl hexosamine uronic acid (22). In addition, the polymer contains up to 15% fatty acids by weight bound in both ester and amide linkages (1). As initially isolated, the bioemulsifier also contains about 15 to 20% (wt/wt) non-covalently bound protein, which can be subsequently removed without affecting emulsifying activity (16, 22). Emulsan initially accumulates on the cell surface of the RAG-1 cell as a minicapsule (4, 12, 13) and is subsequently released into the medium as an active emulsifier when the cells approach the stationary phase (4). In addition, accelerated release of emulsan from the cell surface occurs when early log phase cells are incubated in the presence of inhibitors of protein synthesis such as chloramphenicol. This release of the bioemulsifier depends on the presence of a suitable carbon and nitrogen source in the medium (18). These requirements are presumably due to the de novo synthesis of amino sugar precursors and subsequent polymer synthesis which accompanies release of the emulsan minicapsule. In this report we describe some of the properties of an exocellular esterase activity from RAG-1 which interacts with emulsan. The data indicate that esterase plays a role in the release of emulsan from the cell surface.

MATERIALS AND METHODS

Organisms and culture conditions. The organism used in these studies was *A. calcoaceticus* RAG-1 (ATCC 31012). Cells were grown on ethanol-minimal salts medium containing the following (per liter of water): 22.2 g of $K_2HPO_4 \cdot 3H_2O$, 7.26 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4 g of $(NH_4)_2SO_4$, and 25 ml of absolute ethanol as previously described (4).

Cultures were grown at 30°C for 72 h on a New Brunswick shaker (model G-25) at 250 rpm. Growth was followed turbidometrically with a Klett-Summerson colorimeter equipped with a green filter (1,000 Klett units equals 3.2 g [dry weight] of cells per liter). Dry cell weight was determined by drying samples of washed cell preparations (20 ml) at 80°C to constant weight. Large-scale (1,200-ml working volume) cultures were prepared with a New Brunswick Multigen model F-2000 2-liter fermentor at 30°C. Agitation was 700 rpm, aeration was 600 ml/min, and a pH of 7.0 was maintained automatically with a solution of 2 N NaOH.

Chloramphenicol treatment. Exponential cells were harvested, washed twice with phosphate buffer (0.15 M, pH 7.0), and suspended in the original volume of fresh minimal medium containing 0.8% (vol/vol) ethanol, 1 g of $(NH_4)_2SO_4$ per liter, and 50 μg of chloramphenicol per ml as described previously (18). The suspensions were incubated at 30°C with shaking as described above.

Emulsan assay. Emulsan was assayed functionally on the basis of its ability to form an emulsion of a mixture of hexadecane and 2-methylnaphthalene in 7.5 ml of 20 mM Tris buffer (pH 7.0) containing 10 mM $MgSO_4$ as previously described (4, 16). One unit of emulsan is that amount which gives rise to a turbidity of 100 Klett units in the standard assay. The specific activity of the purified emulsan polymer was 156 U/mg. Emulsan on the cell surface was measured by the immunochemical enzyme-linked immunosorbent assay previously described (4).

Emulsan purification. Emulsan was purified and deproteinized to yield apoemulsan as previously described (22). The final specific activity of the purified apoemulsan was 156 U/mg. Deesterified apoemulsan was prepared by NaOH hydrolysis as previously described (1).

Esterase assay. (i) Hydrolysis of PNP-Ac. The esterase assay is a slight modification of the assay of Higgins and Lapides (6) and Krish (8) and is based on hydrolysis of 4-nitrophenyl acetate (PNP-Ac) to yield 4-nitrophenol (PNP). A sample of enzyme (0.2 ml) was mixed with 1.7 ml of phosphate buffer (75 mM, pH 7.0) containing 10 mM $MgSO_4$, and the reaction was started by the addition of 0.1 ml of 100 mM PNP-Ac in absolute ethanol. Cell-associated enzyme was measured in the same way, except that 0.2 ml of washed cell suspension was substituted for the cell-free preparation. The reaction was run at 30°C and was followed by recording the continuous change in absorbance for 5 min at 405 nm in a Gilford 2400 spectrophotometer with the automatic zero, reference control. The activity was expressed in nanomoles

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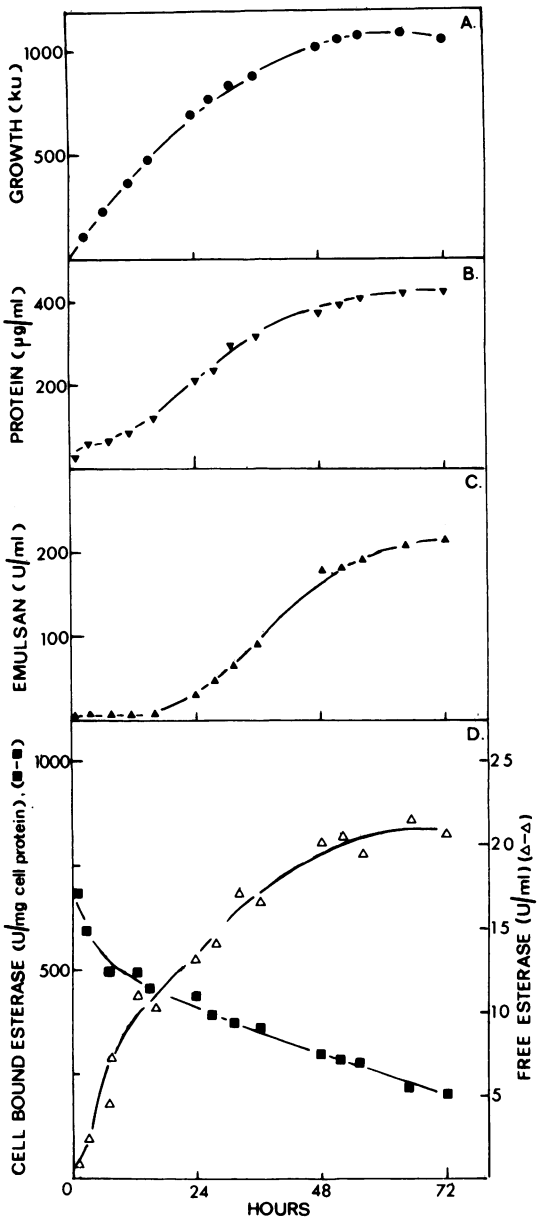

FIG. 1. Esterase distribution during growth of RAG-1. A single colony of RAG-1 was inoculated into 20 ml of ethanol-minimal salts medium and allowed to grow overnight. The inoculum was diluted 1:50 into fresh ethanol-minimal salts medium; samples were removed at the indicated times and assayed for growth (●, A); cell-free protein (▼, B); emulsan activity (▲, C); cell-free esterase (△, D); and cell-bound esterase (■, D). Esterase activity was measured by the PNP-Ac assay as described in the text.

of PNP released (the extinction coefficient of PNP at 405 nm is 9,940 liters per mol per cm). One unit of esterase activity is 1 nmol of PNP per min, and the specific activity is U/mg of protein or, in the case of cell bound enzyme, U/mg of cell protein.

(ii) Hydrolysis of PNP palmitate. When PNP-palmitate was used as a substrate it was first emulsified with sodium deoxycholate and gum Arabic as previously described (20). Spectrophotometric readings of the aqueous phase at 405 nm were made after first separating the emulsion by centrifugation.

(iii) Hydrolysis of triacetin. The reaction was carried out as described above for PNP-Ac hydrolysis, except that the PNP-Ac substrate was replaced by triacetin, and the reaction was carried out for 60 min at 30°C with an enzyme solution of 0.1 mg/ml. Solutions of triacetin in phosphate buffer at the appropriate substrate concentrations were used as controls in the absence of enzyme for each reaction. The acetate released into the medium was assayed directly without extraction from the aqueous medium by gas-liquid chromatography under isothermal conditions at 120°C on a Becker Packard instrument equipped with flame ionization detector and a Spectra Physics Minigrator (model 23000-111). The column was GP 10%, SP 1,200, 1% H_3PO_4 on 80/100 Chromosorb W. Nitrogen was used as a carrier gas. Aqueous solutions of acetic acid ranging from 50 to 200 μg/ml were used as quantitative standards.

Cell-bound esterase. The hydrolysis of PNP-Ac was carried out as described above, except that 0.2 ml of a washed cell suspension (10 to 50 Klett units, corresponding to 25 to 35 μg of cell protein per ml) was used as the enzyme source. The reaction was linear with cell concentrations up to about 10 μg of cell protein per reaction. Specific activity is expressed as units per milligram of cell protein. Protein was assayed by the method of Lowry et al. (10). Cell protein was measured after alkaline treatment (0.2 N NaOH at 100°C for 20 min).

Gel filtration on Sepharose 4B. The emulsan-protein mixture normally present in the cell-free supernatant fluid was precipitated by ammonium sulfate (40% saturation) as described previously (22). The precipitate was dissolved in distilled water and diluted into Tris-hydrochloride buffer (10 mM, pH 7.5) to a final protein concentration of 3 mg/ml. A sample of this solution (5 ml containing about 50 mg of emulsan and 15 mg of protein) was loaded onto a 40- by 2.5-cm Pharmacia K-25 column of Sepharose 4B with a peristaltic pump (Pharmacia Fine Chemicals) at a rate of 12 ml/h. Fractions (5 ml) were eluted at the same rate and collected with an LKB fraction collector. The fractions were analyzed for protein (10), amino sugars (3), emulsifying activity, and esterase (using the standard PNP-Ac hydrolysis assay).

DEAE-Sephacel chromatography of esterase and apoemulsan. An apoemulsan solution (3 ml; 1 mg/ml) in 10 mM Tris-hydrochloride buffer (pH 8.0), was loaded onto a column of DEAE-Sephacel (12 by 1.5 cm; column bed volume, 20 ml) and the column was washed with 30 ml of the same buffer. Two milliliters of a solution of purified esterase obtained by gel filtration on Sepharose 4B containing 150 μg of protein per ml (specific activity, 250 U/mg of protein) was then loaded onto the column, and the column was once again washed with 30 ml of loading buffer. The emulsan was then eluted by applying a linear gradient between 0 and 0.35 M KCl in 10 mM Tris-hydrochloride buffer (pH 8.0). Fractions of 1 ml were collected and assayed for both emulsan and esterase activity. The procedure was repeated with deesterified apoemulsan in place of the fully active emulsifier. In the absence of preloading with emulsan, esterase did not bind to the column.

RESULTS

Release of esterase during the growth cycle. The kinetics of RAG-1 growth, emulsan production, and esterase appearance in the cell-free growth medium are shown in Fig. 1. During the first 12 h there was a rapid release of esterase and a slow release of protein into the growth medium, accompanied by a decrease in cell-bound enzyme activity. Cell-free esterase activity continued to rise slowly and continuously for the next 36 h. The production of emulsan began at about 18 h and was accompanied by a corresponding release of proteins. By 48 h, about 210 U of emulsan activity per ml (1.3 g/liter) and 400 μg of protein per ml were found in the

cell-free supernatant. Between 18 and 30 h there was a rapid decrease in extracellular esterase specific activity because of the differential rate of release of esterase (from 10 to 14 U/ml) and protein (from 75 to 375 μg/ml) during that period. By about 36 h, cell-free esterase had achieved a constant specific activity about 50 U/mg of protein.

Effect of chloramphenicol on release of emulsan and esterase. In addition to its release into the cell-free medium during the growth cycle of RAG-1, emulsan has also been shown to be released from the cells under conditions of protein synthesis inhibition by either amino acid starvation or chloramphenicol (4, 18). This chloramphenicol-mediated release required both a carbon and a nitrogen source. It was of interest, therefore, to examine the effect of chloramphenicol on the release of esterase from the cell surface of RAG-1 (Fig. 2). RAG-1 cells were exposed to 50 μg of chloramphenicol per ml in the presence or absence of either a carbon source, a nitrogen source, or both, and the culture was monitored for (i) cell mass (Fig. 2A), (ii) cell-free emulsan (Fig. 2B), (iii) cell-bound esterase (Fig. 2C), and (iv) cell-free esterase (Fig. 2D). In confirmation of previous results (18), emulsan was released in the presence of chloramphenicol only when the medium was supplemented with both a carbon and a nitrogen source. In contrast, starvation for carbon was sufficient to bring about chloramphenicol-mediated esterase release from the cell surface (Fig. 2C and D). When starved for nitrogen, the cells retained the same high level of cell-bound esterase (500 U/mg of cell protein), and no enzyme appeared in the supernatant fluid; in the complete system emulsan release proceeded almost linearly and continuously for the entire 6-h treatment, and over 90% of the released esterase was released within the first hour. As expected, once esterase was released, chloramphenicol prevented the resynthesis of the enzyme, even in the complete system containing both carbon and nitrogen (Fig. 2C).

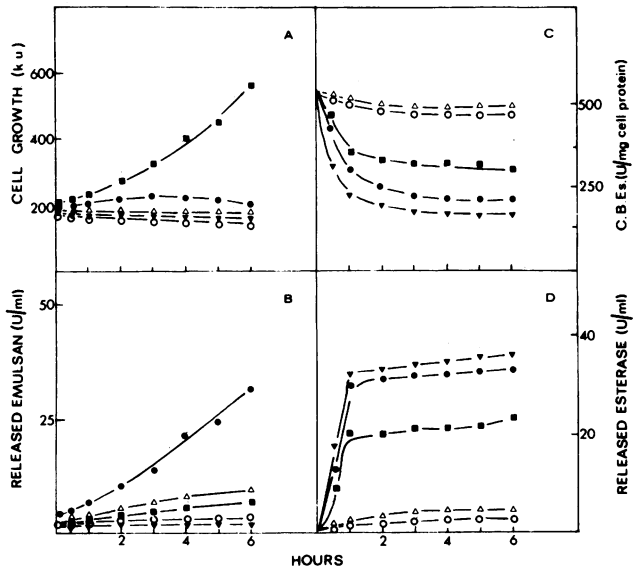

FIG. 2. Release of esterase and emulsan from the cell surface of RAG-1 in the presence of chloramphenicol. An exponential culture of RAG-1 (500 ml) was prepared as described in the text. After washing, the culture was divided into five equal cultures, each of which was suspended in minimal salts medium containing the following: no additions (■), chloramphenicol (●), chloramphenicol without ethanol (▼), chloramphenicol without $(NH_4)_2SO_4$ and with ethanol (△), or chloramphenicol without ethanol and without $(NH_4)_2SO_4$ (○). Ethanol was added at a concentration of 0.8% (vol/vol), and $(NH_4)_2SO_4$ was added at 4 g/liter. Samples from each culture were removed at the indicated times and assayed for turbidity (A), cell-free emulsan activity (B), cell-bound esterase (C), and cell-free esterase (D) as described in the legend to Fig. 1.

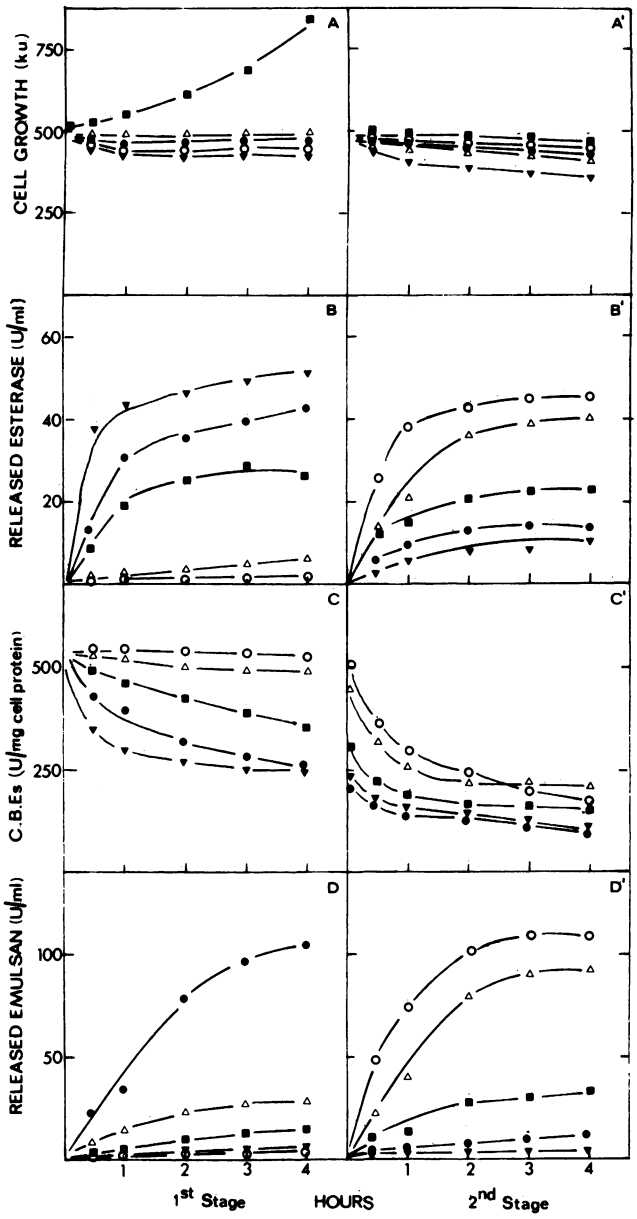

FIG. 3. Release of esterase and emulsan after pretreatment of RAG-1 cells in the presence of chloramphenicol. Five exponential cultures of RAG-1 were incubated as described in the legend to Fig. 2. At various times, samples were removed and assayed for growth (A and A'), cell-free esterase (B and B'), cell-bound esterase (C and C'), and cell-free emulsan (D and D'). After 4 h of incubation, each culture was harvested and suspended in complete ethanol-minimal salts medium containing 50 μg of chloramphenicol per ml (A', B', C', and D'). At various times samples were removed and assayed as described in the legend to Fig. 2. Each culture was pretreated separately in minimal salts medium containing the following: no additions (■), chloramphenicol (●), chloramphenicol without ethanol (▼), chloramphenicol without $(NH_4)_2SO_4$ (△), or chloramphenicol without ethanol and without $(NH_4)_2SO_4$ (○). The symbols in A', B', C', and D' refer to the pretreatment conditions before suspending in complete ethanol-minimal salts-chloramphenicol medium.

The fact that esterase could be released from the cell surface in the presence of chloramphenicol without the concomitant release of emulsan suggested a way to study the

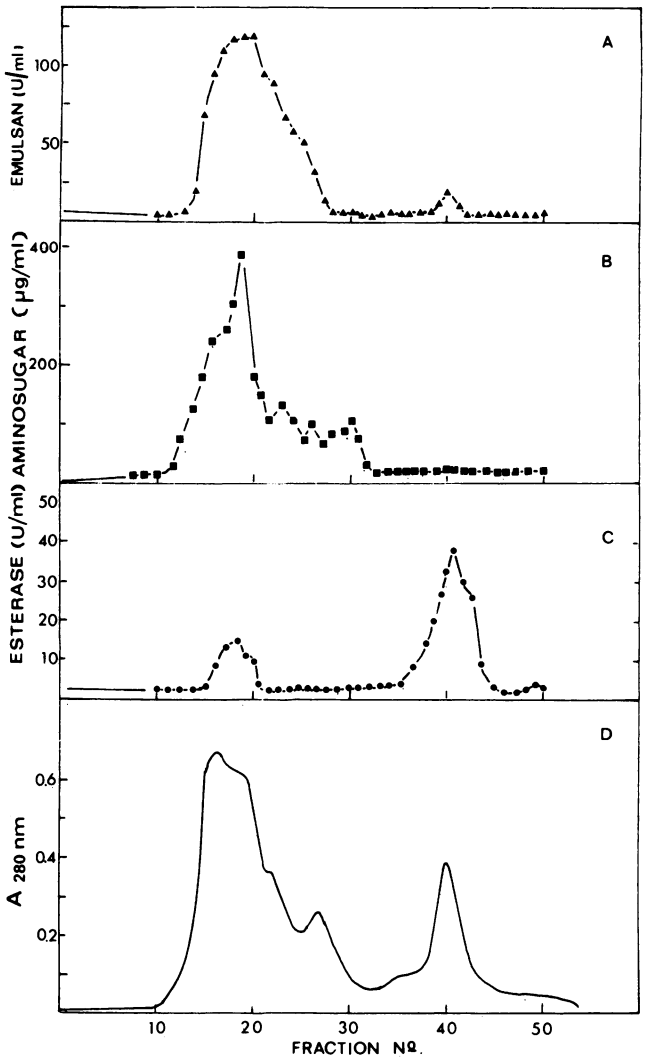

FIG. 4. Gel filtration on Sepharose 4B. An ammonium sulfate (40% saturation) precipitate of the cell-free supernatant from a 72-h culture was loaded, after dialysis, onto a 40- by 2.5-cm Sepharose 4B column and eluted with 10 mM Tris buffer (pH 7.5). The eluate was continuously monitored for UV absoption at 280 nm (D). Fractions were assayed for emulsan activity (A), amino sugars (B) (4), and esterase activity (PNP-Ac hydrolysis) (C).

possible involvement of esterase in emulsan release. It was of interest to determine whether cells from which esterase had previously been released in the presence of chloramphenicol, but in the absence of a carbon source, could subsequently release emulsan in a complete chloramphenicol system in which no new esterase could be synthesized (Fig. 3). Five different cultures were prepared and exposed to chloramphenicol under different conditions as described above (Fig. 3A, B, C, and D). Each culture was then washed and suspended in the presence of chloramphenicol along with a carbon and a nitrogen source (Fig. 3A', B', C', and D'). Cells from which about 50% of the cell-bound esterase had been released in the first stage (Fig. 3B and C) no longer released emulsan in the second stage (Fig. 3D'). In contrast, cells that were first incubated in the presence of chloramphenicol in the absence of a nitrogen source did not release either esterase or emulsan into the medium (Fig. 3B, C, and D). However, when such cells were suspended in the presence of a complete chloramphenicol system, up to 100 U of emulsan per ml was released into the medium within 2 h. The results indicate that, in addition to the presence of a carbon and nitrogen source, the process of emulsan release requires the presence of an active esterase on the cell surface.

Gel filtration of esterase. After coprecipitation of emulsan and cell-free esterase activity in 40% saturated ammonium sulfate, the dialyzed suspension was chromatographed on a Sepharose 4B column as described above (Fig. 4). The protein mixture eluted in three major peaks. The first peak appeared in the void volume simultaneously with both emulsan activity and a peak of esterase activity, a second protein peak followed shortly, and a third protein peak appeared much later. This last protein fraction, which was free of emulsan, contained the bulk of the esterase activity. About 87% of the total protein and 93% of the emulsan activity were recovered in the three protein fractions. The specific activity of the esterase increased from 22 to 219 U/mg of protein after gel filtration. The fractions containing the bulk of the esterase (fractions 39, 40, and 41 in Fig. 4)

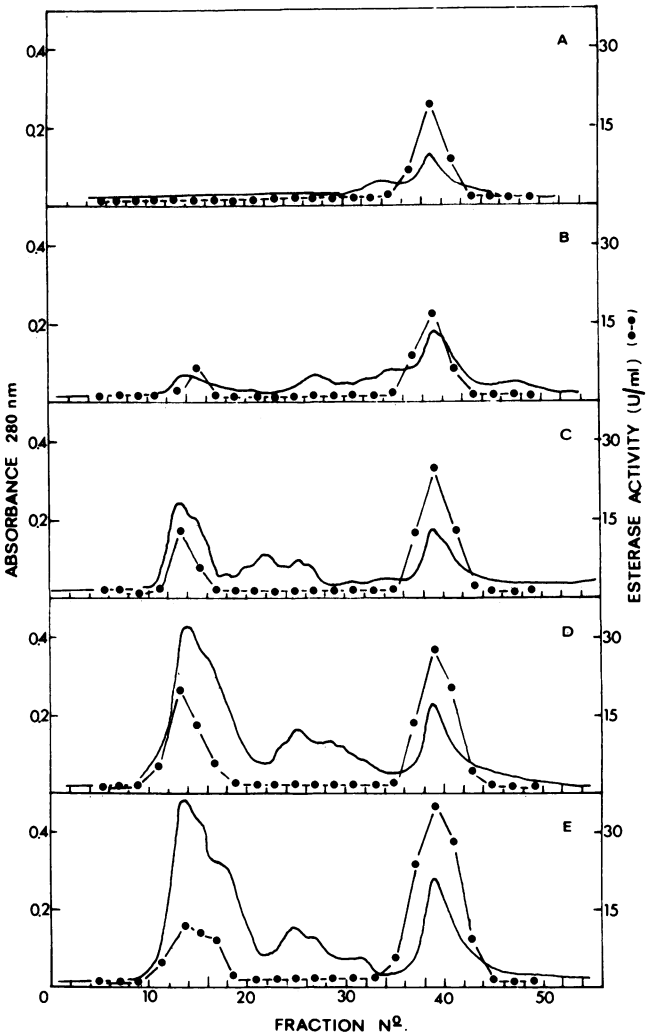

FIG. 5. Distribution of free esterase and emulsan-associated esterase during growth of RAG-1. RAG-1 was grown in a 2-liter fermentor as described in the text. Samples (50 ml) were removed at 1 h (A), 4 h (B), 8 h (C), 16 h (D), and 32 h (E) and centrifuged, and a crude preparation of esterase was prepared by precipitation with ammonium sulfate (40% saturation). Samples were chromatographed on Sepharose 4B columns as described in the text. Absorbance at 280 nm was monitored continuously, and esterase was measured by the PNP-Ac assay.

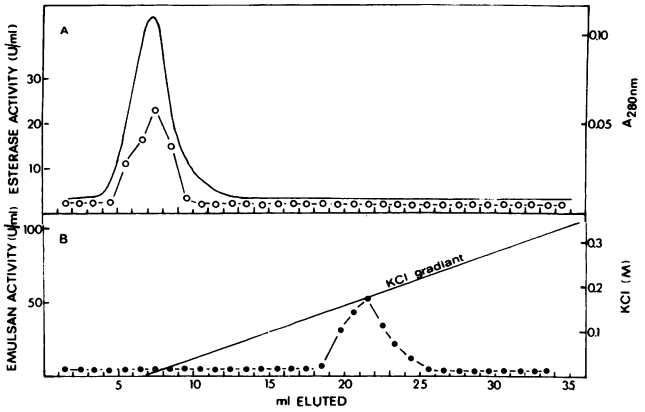

FIG. 6. Chromatography of esterase on DEAE-Sephacel preadsorbed with deesterified apoemulsan. The chromatography was carried out as described in the text. Apoemulsan was first deesterified by base hydrolysis (0.1 N NaOH, 100°C, 30 min) before preadsorption. The column was eluted with a linear KCl gradient (0 to 0.35 M). Absorbance at 280 nm was monitored continuously. Esterase (○) was measured by the PNP-Ac assay, and emulsan (●) was determined using the standard emulsification assay.

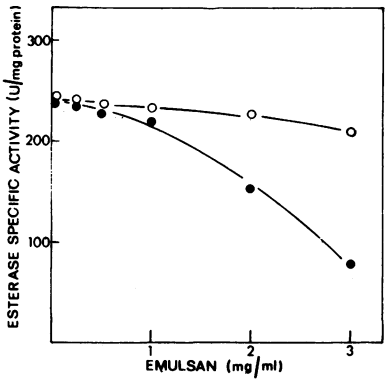

FIG. 7. Inhibition of esterase activity by apoemulsan and deesterified apoemulsan. Samples of esterase partially purified by gel filtration were assayed by using PNP-Ac in the presence of increasing concentrations of apoemulsan (●) or deesterified apoemulsan (○).

were pooled from several chromatographic runs and used in the characterization of the partially purified esterase.

Distribution of free and emulsan-associated esterase. To determine how the distribution of free esterase and emulsan-associated cell-free esterase varied throughout the growth cycle (Fig. 5), the kinetics of enzyme appearance in the cell-free medium was determined by examining 50-ml samples taken from a 1.2-liter culture of RAG-1 grown in a fermentor under controlled conditions (see above). The cell-free supernatant fluids were obtained from each sample; after precipitation with 40% saturated ammonium sulfate and dialysis, each of the crude preparations was chromatographed on a Sepharose 4B column as described above. During the early stages of growth (Fig. 5A and B), the vast majority of enzyme appeared as free esterase. However, by 8 h (Fig. 5C), emulsan-associated esterase constituted a sizeable fraction of the total cell-free enzyme. By 32 h the emulsan-associated esterase began to decrease, whereas the free form of the enzyme increased significantly. After 32 h the low level of emulsan-associated esterase remained constant (data not shown).

Esterase substrates. The partially purified esterase also catalyzed the hydrolysis of acetyl groups from a number of substrates, including triacetin and even emulsan itself (data not shown). In these cases, the maximal rate of hydrolysis was only about 1/10 the rate observed with PNP-Ac. In addition, the enzyme also exhibited lipolytic activity in the hydrolysis of PNP-palmitate, although the maximal rate of hydrolysis was some 50-fold lower than with PNP-Ac (data not shown).

Interactions of esterase and emulsan. The affinity of esterase for emulsan was demonstrated by using DEAE-Sephacel to which apoemulsan had previously been adsorbed. When the enzyme was loaded onto the DEAE-Sephacel column in the absence of apoemulsan, more than 95% of the activity was recovered in the void volume, indicating that the enzyme did not bind to the column. However, when the enzyme was run on the same column after prior adsorption of apoemulsan, the elution profile changed dramatically, and the enzyme activity eluted only in the presence of a KCl gradient simultaneously with the emulsifier. When the same experiment was carried out with preadsorbed, deesterified apoemulsan (Fig. 6), the enzyme appeared in the wash buffer, whereas the preadsorbed deesterified polymer was eluted with the KCl gradient. The overall recoveries of the chromatography are shown in Table 1. Of interest was the finding that the specific activity of the emulsan-free enzyme was 240 U/mg of protein, whereas the specific activity of the apoemulsan-associated material was 146 U/mg of protein, even though 96% of the input protein was recovered with the esterase. Further evidence that esterase is partially inhibited as a result of its interaction with emulsan is presented in Fig. 7. It should be noted that this inhibition did not occur with the deesterified bioemulsifier.

DISCUSSION

The results presented in this report indicate a role for cell-bound esterase in the release of emulsan from the

TABLE 1. Chromatography of esterase on DEAE-Sephacel[a]

Preadsorption[b]	Process	Emulsan (U)	Recovery (%)	Esterase (U)	Recovery (%)	Protein (μg)	Recovery (%)	Esterase (U/mg of protein)
None	Loaded			72		300		240
	Eluted			66	92	280	95	240
Apoemulsan	Loaded	438		72		300		240
	Eluted	417	95	42	58	290	96	150
Deesterified	Loaded	204		72		300		240
apoemulsan	Eluted	192	95	68	94	290	97	240

[a] A sample of partially purified esterase (Fig. 4) was loaded onto a DEAE-Sephacel column and eluted with a linear KCl gradient (0 to 0.35 M) as described in the legend to Fig. 6. Emulsan was measured by the standard emulsification assay, and esterase activity was measured by the PNP-Ac assay. Values for eluted esterase and emulsan refer to the pooled active fractions.

[b] The column was preloaded with either 2 mg of apoemulsan or deesterified apoemulsan (Fig. 6) washed, and loaded with 300 μg of esterase.

surface of strain RAG-1. The basis of this conclusion stems from studies on the release of emulsan and esterase in the presence of chloramphenicol. In the absence of a carbon source, the esterase was released, whereas emulsan was retained on the cell surface (Fig. 2B). Once esterase was removed, the emulsan was no longer released in a complete chloramphenicol system supplemented with a carbon source. It remains to be determined why the release of esterase requires the presence of a nitrogen, but not necessarily a carbon source. When washed cells pregrown on unlabeled ethanol were exposed to chloramphenicol together with [^{14}C]ethanol and a nitrogen source such as ammonia, ^{14}C-labeled emulsan was released into the medium (18). It thus appears that in the case of chloramphenicol-mediated emulsan release, the requirement for a nitrogen source may be caused by a requirement for amino sugar emulsan precursor synthesis and the coupling of de novo emulsan synthesis to bioemulsifier release (18).

In a 72-h growth experiment the total yield per milliliter of extracellular esterase activity was about 15 U at the end of 72 h (38 U/mg of protein × 0.4 mg of extracellular protein), whereas during the same time period there was still 480 U associated with the cells (280 U/mg of cell protein × 2 mg of cellular protein per ml). Nevertheless, although only a small portion of cell-associated esterase was released either in the complete chloramphenicol system or in the absence of a carbon source, this release of esterase was sufficient to prevent subsequent emulsan release. It may be that only a fraction of esterase specifically associated with emulsan at the cell surface is involved in the release of the bioemulsifier. An alternative explanation is that there is more than a single esterase (or lipase) and that only one of these is involved in emulsan release. We favor the first hypothesis, since point mutants defective in esterase activity appear to be defective in extracellular emulsan production; these mutations are also affected in cell-bound esterase (Y. Shabtai, manuscript in preparation). Since emulsan was shown to be a substrate for the esterase enzyme, it is tempting to suggest that the release process involves the cleavage of an ester bond involved in the association of emulsan with the cell surface.

Accelerated release in the presence of chloramphenicol of a lipopolysaccharide-phospholipid protein complex from the outer membranes of *Escherichia coli* and *Salmonella typhimurium* has been reported previously (17). This complex appeared to contain only a single protein species.

An emulsan esterase complex was also formed in vitro with a DEAE affinity column in which adsorbed apoemulsan was used selectively to bind the cell-free esterase. It is of interest that deesterified emulsan was not effective in enzyme binding. Experiments are currently in progress employing this approach to isolate other RAG-1 extracellular proteins that bind specifically to active emulsan.

Another role for esterase may involve the growth of RAG-1 on triglycerides, which must first be degraded to fatty acids to serve as carbon sources (glycerol itself is not a carbon source for RAG-1). The fact that a class of RAG-1 mutants unable to grow on triacetin was also defective in esterase activity (Y. Shabtai, in preparation) supports this hypothesis.

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